

Identification of Triterpenoids and Flavonoids from the Seeds of Tartary Buckwheat[†]

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Abstract – Phytochemical constituents were isolated from the seeds of tartary buckwheat (*Fagopyrum tataricum*) by open column chromatography. Their structures were elucidated as β -sitosterol (**1**), β -sitosterol-3-*O*-glucoside (**2**), oleanolic acid (**3**), kaempferol (**4**), quercetin (**5**), kaempferol-3-*O*-rutinoside (**6**), and quercetin-3-*O*-rutinoside (**7**) on the basis of spectroscopic analysis including ¹H-, ¹³C-NMR, and MS. To our knowledge, oleanolic acid (**3**) has been isolated for the first time from the seeds of *Fagopyrum* species. The total contents of compounds **4** - **7** were 0.500 mg/g in Daesan maemil, 0.312 mg/g in Yangjul maemil, and 2.185 mg/g in tartary buckwheat.

Keywords – *Fagopyrum tataricum*, Oleanolic acid, Flavonoid, Quantitative analysis

Introduction

The genus *Fagopyrum* (Polygonaceae) is mostly distributed in the North Temperate Zone. There are many species of buckwheat, and 9 main buckwheat species have agricultural value. Generally, 2 types are used around the world: common buckwheat (*Fagopyrum esculentum*) and tartary buckwheat (*F. tataricum*). Common buckwheat originates from southwest China, while tartary buckwheat (TB) is grown and used in the mountainous regions of northern India, Bhutan, and Nepal (Christa and Soral-Smietana, 2008). TB is characterized by bitter taste, small seeds, and a tight seed coat that makes dehulling difficult. TB is also reported to contain more rutin in the seeds than common buckwheat (Steadman *et al.*, 2001; Fabjan *et al.*, 2003). TB is important as a pharmaceutical plant, used for antioxidant and antitumor properties. It also reduces blood cholesterol levels and regulates blood sugar levels, blood lipid levels, and blood pressure in the human body (Ren *et al.*, 2001; Tomotake *et al.*, 2006; Guo *et al.*, 2007; Liu *et al.*, 2008; Yao *et al.*, 2008; Mok *et al.*, 2011; Lee *et al.*, 2011a; Choi *et al.*, 2012; Choi *et al.*, 2013). The root of TB is an important medicinal

substance, which was traditionally used in folk medicine to treat chronic diseases, such as cancers, rheumatic disorders, and general debility (Guo *et al.*, 2006).

The major chemical components of TB are the abundant flavonoids and phenylpropanoids (Li *et al.*, 2001; Zheng *et al.*, 2012). TB products are important functional foods and are also rich in vitamins B1, B2, and B6; various essential proteins and trace elements; dietary fibers; and D-*chiro*-inositol (Kreft *et al.*, 1999; Steadman *et al.*, 2000; Zielinski and Kozłowska, 2000; Wang *et al.*, 2009). Analysis of buckwheat has shown the presence of phenolic components (chlorogenic acid, orientin, isoorientin, vitexin, isovitexin, rutin, and quercetin) in common buckwheat and TB sprouts (Kim *et al.*, 2008) and of resveratrol in the grains, hulls, and leaves of common buckwheat and TB (Němcová *et al.*, 2011). A time-course study has been also performed on flavonoids in TB sprouts (Kim *et al.*, 2007).

To date, many studies have described the isolation and identification of compounds from *F. esculentum*; however, investigations into TB seeds have been scarce. This research was therefore carried out to focus on the isolation and identification of compounds from TB seeds using open column chromatography.

Experimental

Plant materials – The seeds of TB (*F. tataricum*;

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Polygonaceae) were provided by Highland Agriculture Research Center, RDA, Korea.

Instruments and reagents – Electron ionization mass spectra (EI-MS) were performed using a JEOL JMS-600W (Tokyo, Japan) mass spectrometer. ^1H - and ^{13}C -nuclear magnetic resonance (NMR) spectra were recorded with a Bruker AVANCE 300 or 500 NMR (Rheinstetten, Germany) spectrometer with CDCl_3 , pyridine- d_5 , or $\text{DMSO-}d_6$ using TMS as an internal standard. Chemical shifts has been reported in parts per million (δ), and coupling constants (J) have been expressed in Hertz. The TLC analysis was conducted with Kiesel gel 60 F254 plates (silica gel, 0.25 mm layer thickness; Art. 5715, Merck Co., Germany), with compounds visualized by spraying with 10% H_2SO_4 followed by charring at 60 °C. Open column chromatography was performed using a silica gel (200 - 400 mesh; Merck Co., Germany) and Sephadex LH-20 (25 - 100 μm ; Amerham Biosciences, Sweden). Simultaneous analysis was performed with a Agilent 1100 series system (Waldbronn, Germany) equipped with a G1311A Quat-pump and an a G1315B DAD detector. The water and acetonitrile used in this research were of HPLC grade, and all the other reagents were of analytical grade.

Extraction and isolation – The dried whole seeds of TB (9995.2 g) were ground into powder, and extracted with MeOH (10 L \times 3) under reflux conditions. The resultant extracts were combined and concentrated under reduced pressure to afford 399.8 g of the residue. The MeOH extract was suspended in water and then partitioned successively with equal volumes of *n*-hexane (54.2 g), CH_2Cl_2 (15.9 g), EtOAc (6.1 g) and *n*-BuOH (21.1 g). A portion of the *n*-hexane and CH_2Cl_2 fractions was subjected to chromatography on a silica gel column eluting with a gradient of *n*-hexane-EtOAc (90 : 10, 70 : 30) and EtOAc-MeOH (95 : 5) to afford compounds 1 - 3. A portion of the EtOAc fraction was subjected to chromatography on a silica gel column and eluted with a gradient of CH_2Cl_2 -MeOH (100% CH_2Cl_2 up to 80% MeOH) to afford 7 subfractions (E_1 - E_7). Subfraction E_2 (CH_2Cl_2 : MeOH = 95 : 5) was conducted by rechromatographed on sephadex LH-20 eluting with MeOH to obtained through a second round of chromatography on Sephadex LH-20, which was eluted with MeOH to afford compound 4; the subfraction E_3 (CH_2Cl_2 : MeOH = 85 : 15) was obtained through a second round of chromatography on Sephadex LH-20, which was eluted with MeOH to afford compound 5. A portion of the *n*-BuOH fraction was subjected to chromatography on a silica gel column, which was eluted with a gradient of CH_2Cl_2 -MeOH (100%

MeOH) to afford 8 subfractions (B_1 - B_8). Subfraction B_3 (CH_2Cl_2 : MeOH = 85 : 15) was obtained through a second round of chromatography on Sephadex LH-20, which was eluted with MeOH to afford compound 6; subfraction B_5 (CH_2Cl_2 : MeOH = 60 : 40) was obtained through a second round of chromatography on Sephadex LH-20, which was eluted with MeOH to afford compound 7.

Compound 1 – white powder; EI-MS m/z : 414 $[\text{M}]^+$ (100), 396 (42.5), 381 (21.8), 329 (25.0), 303 (28.9), 289 (4.0), 273 (25.3), 255 (48.0), 231 (15.9), 213 (25.2), 159 (25.6), 145 (25.8); ^1H - and ^{13}C -NMR (300 MHz, CDCl_3): see Table 1.

Compound 2 – white powder; FAB-MS m/z : 577 $[\text{M} + \text{H}]^+$; ^1H - and ^{13}C -NMR (300 MHz, pyridine- d_5): see Table 1.

Compound 3 – white powder; EI-MS m/z : 456 $[\text{M}]^+$ (2.5), 438 (1.6), 423 (0.9), 410 (1.2), 248 (100), 207 (27.5), 203 (40.5); ^1H - and ^{13}C -NMR (500 MHz, CDCl_3): see Table 1.

Compound 4 – yellow powder; EI-MS m/z : 286 $[\text{M}]^+$ (100), 258 (8.8), 229 (6.2), 153 (3.2), 143 (5.5) 121 (8.0); ^1H - and ^{13}C -NMR (500 MHz, $\text{DMSO-}d_6$): see Table 2.

Compound 5 – yellow powder; EI-MS m/z : 302 $[\text{M}]^+$ (100), 274 (9.8), 245 (7.2), 228 (7.4), 153 (7.5), 137 (8.2), 128 (8.0), 69 (4.7); ^1H - and ^{13}C -NMR (500 MHz, $\text{DMSO-}d_6$): see Table 2.

Compound 6 – yellow powder; FAB-MS m/z : 595 $[\text{M} + \text{H}]^+$; ^1H - and ^{13}C -NMR (500 MHz, $\text{DMSO-}d_6$): see Table 2.

Compound 7 – yellow powder; FAB-MS m/z : 611 $[\text{M} + \text{H}]^+$; ^1H - and ^{13}C -NMR (500 MHz, $\text{DMSO-}d_6$): see Table 2.

Sample preparation – We used 10.0 g seeds to analyze the flavonoid content (compounds 4 - 7) in 3 *Fagopyrum* species. The seeds from Daesan maemil (DM), Yangjul maemil (YM), and TB were extracted with 100% MeOH (100 ml) 3 times under reflux conditions and the solvent was evaporated *in vacuo*. The residue was dissolved in 1 mL of MeOH and then sonicated for 10 min. The resultant solution was filtered with a Whatman 0.2 μm nylon syringe filter. The resulting solution was used for HPLC analysis.

HPLC conditions – A Discovery[®] C18 (4.6 \times 250 mm, 5 μm) column was used for simultaneous analysis of the flavonoids (compounds 4 - 7). The mobile phase was acetonitrile (solvent A) and 0.4% phosphoric acid (solvent B). The gradient elution program decreased solvent B from 90% to 80% for 5 min and then to 50% for 35 min. The injection volume was 10 μL and the flow rate was 1 mL/min. The UV chromatograms were recorded at 350

Table 1. ^1H - and ^{13}C -NMR spectral data for compounds **1** - **3**

No.	1		2		3	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}
1		37.4		37.3		38.9
2		31.8		29.4		28.0
3	3.52 m	71.9		78.5	3.65 dd (6.0, 12.6)	78.2
4	2.27 m	42.4		38.4		39.5
5		141.0		141.3		55.8
6	5.35 m	121.9	5.35 m	122.3		18.9
7		32.0		31.8		33.4
8		32.0		30.6		39.9
9		50.3		50.7		48.3
10		36.7		36.8		37.4
11	1.99 m	21.2		20.4		23.9
12		39.8		40.3	5.26 t (3.5)	122.7
13		42.4		42.7		144.9
14		57.0		56.9		42.3
15		24.5		23.8		28.3
16		28.4		26.7		23.9
17		56.1		56.6		46.9
18	0.68 s	12.0	0.67 s	12.4	3.22 dd (4.8, 11.1)	46.6
19	1.01 s	19.1	0.94 s	19.6		42.2
20		36.3		34.5		31.0
21	0.92 d (6.3)	18.9	1.00 d (6.3)	19.4		34.4
22		34.1		32.6		33.3
23		26.2		24.9	0.99 s	28.9
24		46.0		46.4	0.88 s	16.6
25		29.1		28.9	0.78 s	15.7
26	0.81 d (6.3)	19.1	0.94 d (5.4)	19.8	0.93 s	17.6
27	0.86 d (4.2)	19.6	0.89 d (6.3)	20.1	1.09 s	26.3
28		23.2		21.7		180.1
29	0.80 t (5.8)	12.1	0.89 m	12.5	0.80 s	33.4
30					0.89 s	23.9
Glc-1			5.09 d (6.9)	103.0		
2				75.8		
3				79.0		
4				72.1		
5				78.9		
6				63.2		

Chemical shifts are reported in parts per million (δ) and coupling constants (J) are expressed in Hertz. Compounds **1** and **2**: 300 MHz, Compound **3**: 500 MHz

nm for quantification of the flavonoids. All the injections were performed in triplicate.

Calibration curve – A stock solution (1 mg/mL) of each flavonoid isolated from seeds of TB was prepared in MeOH, successively reducing the solution content to 50% to obtain different concentrations. The content of each analyte was then determined from the corresponding calibration curves. The calibration functions of the

flavonoids were calculated using the peak area (Y), concentration (X , $\mu\text{g}/10 \mu\text{L}$), and mean ($n = 5$) \pm standard deviation values.

Results and Discussion

The seeds of TB were subjected to extraction with MeOH and successive partitioning with *n*-hexane,

Table 2. ^1H - and ^{13}C -NMR spectral data for compounds **4** - **7** (500 MHz)

No.	4		5		6		7	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}
2		146.8		145.0		156.0		156.4
3		135.6		135.7		132.6		133.3
4		175.9		175.8		176.7		177.3
5		160.7		161.0		160.6		161.2
6	6.18 (d, 1.5)	99.4	6.18 (d, 2.0)	98.2	6.18 (d, 2.0)	98.3	6.19 (d, 2.0)	98.7
7		163.9		164.0		164.2		164.1
8	6.43 (d, 1.5)	93.5	6.40 (d, 2.0)	93.3	6.38 (d, 2.0)	93.2	6.38 (d, 2.0)	93.6
9		158.1		156.1		156.1		156.6
10		103.0		103.0		103.2		103.9
1'		123.6		121.9		120.3		121.1
2'	8.04 (d, 8.5)	129.5	7.67 (d, 2.0)	120.0	7.98 (dd, 2.0, 7.0)	130.3	7.53 (d, 2.1)	121.6
3'	6.92 (d, 8.5)	115.4		148.0	6.88 (dd, 2.0, 7.0)	114.5		144.8
4'		159.2		147.0		159.3		148.4
5'	6.92 (d, 8.5)	115.4	6.88 (d, 8.5)	115.6	6.88 (dd, 2.0, 7.0)	114.5	6.83 (d, 8.0)	116.2
6'	8.04 (d, 8.5)	129.5	7.53 (dd, 2.0, 8.5)	115.0	7.98 (dd, 2.0, 7.0)	130.3	7.53 (dd, 2.1, 8.0)	115.2
5-OH	12.47 (s)		12.48 (s)		12.54 (s)		12.59 (s)	
Glc-1					5.30 (d, 7.5)	100.9	5.34 (d, 7.2)	101.2
2						73.7		74.0
3						75.2		75.9
4						69.4		70.0
5						75.9		76.4
6						66.4		67.0
Rha-1					4.38 (d, 1.0)	100.2	4.38 (br s)	100.7
2						69.8		70.3
3						70.1		70.5
4						71.3		71.8
5						67.7		68.2
6					0.99 (d, 6.0)	17.2	0.99 (d, 6.2)	17.7

Same as in Table 1.

Table 3. Calibration curves for compounds **4** - **7**

Compound	t_{R}	Calibration equation ^a	Correlation factor, r^2 ^b
4	22.9	$Y = 4,191.2677X - 0.7537$	0.9999
5	18.2	$Y = 1,047.8796X - 0.3583$	0.9997
6	10.3	$Y = 1,105.7458X + 7.6905$	0.9996
7	11.9	$Y = 728.2972X + 12.9911$	0.9995

^a Y = peak area, X = concentration of the standard ($\mu\text{g}/\text{mL}$).

^b r^2 = correlation coefficient for 3 data points in the calibration curve ($n = 5$).

CH_2Cl_2 , EtOAc, and *n*-BuOH. Each fraction was used for sequential open column chromatography over silica gel to obtain compounds **1** - **7**.

Compounds **1** and **2** were obtained as white powders from the *n*-hexane and CH_2Cl_2 fractions. The ^1H -NMR spectra of **1** and **2** showed the existence of a sterol

skeleton. The olefinic proton broad doublet one signal was observed at δ 5.35 (H-6). The 2 angular methyl singlets of H-18 and -19 at δ 0.67-0.68 and 0.94-1.01, respectively, and the 3 doublets of H-21, -26, and -27 at δ 0.92-1.00, 0.81-0.94 and 0.86-0.89, respectively, were identified. The ^{13}C -NMR spectra of **1** and **2** showed 27

Table 4. Contents of compounds **4 - 7** in the seeds of *Fagopyrum* species

Sample	Content (mg/g)				Total
	4	5	6	7	
DM	–	0.018 ± 0.001	0.033 ± 0.002	0.450 ± 0.004	0.500 ± 0.007
YM	–	0.013 ± 0.001	0.032 ± 0.001	0.266 ± 0.009	0.312 ± 0.009
TB	0.007 ± 0.001	0.055 ± 0.005	0.082 ± 0.005	2.040 ± 0.166	2.185 ± 0.172

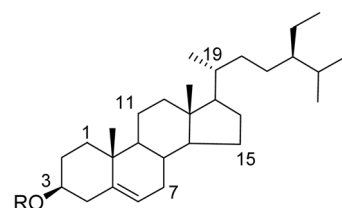
Data are the mean ± S.D. (n = 4) values in terms of mg/g of the dried samples.

–: Not determined.

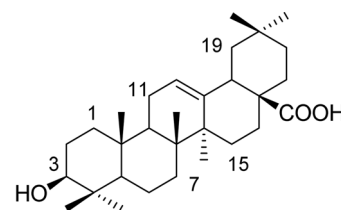
and 35 resonances, respectively. The C-5 and -6 signals of **1** and **2** were identified at δ 141.0-141.3 and 121.9-122.3, respectively. Compounds **1** and **2** had similar structural signals; however, the typical pattern of a glucose moiety of **2** was shown in the ^1H - and ^{13}C -NMR spectra. The anomeric proton of glucose of **2** was detected by HMBC analysis at δ 5.09 (d, $J=6.9$ Hz); and glucose was detected at C-3 (β -linkage) of aglycone. Then, the structures of **1** and **2** were elucidated as β -sitosterol and β -sitosterol-3-*O*-glucoside (daucosterol), respectively, through a comparison of the spectral data described in the literature (Bao *et al.*, 2003; Lee *et al.*, 2011b).

Compound **3** was obtained as a white powder from the *n*-hexane fraction. It showed a molecular ion peak at m/z 456 $[\text{M}]^+$ in the EI-MS analysis. The ^1H -NMR spectrum of **3** showed a terpenoid feature. In the ^1H -NMR spectra, 1 olefinic proton signal at δ 5.26 (H-12), 1 oxygen bearing methine proton signals at δ 3.65 (H-3), and 7 methyl proton signals at δ 0.78 ~ 1.09 were identified. In the ^{13}C -NMR spectra of **3**, olefinic carbon signals of C-12 and -13 at δ 122.7 and 144.9, respectively, and 1 ester carboxyl group signal of C-28 at δ 180.1 were observed. The oxygen bearing carbon signal of C-3 showed at δ 78.2. The structure of **3** was elucidated as oleanolic acid through a comparison of the spectral data described in the literature (Gohari *et al.*, 2009).

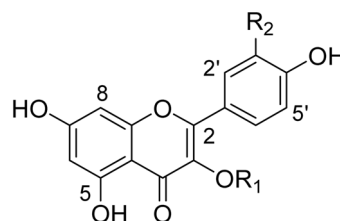
Compounds **4 - 7** were obtained as yellow powder from the EtOAc and *n*-BuOH fractions. The ^1H -NMR spectra showed a characteristic proton signal at δ 12.47-12.59 corresponding to a free hydroxy group at C-5. The meta coupling signal at δ 6.18 (d, $J=1.5$ Hz) and 6.43 (d, $J=1.5$ Hz), δ 6.18 (d, $J=2.0$ Hz) and 6.40 (d, $J=2.0$ Hz), δ 6.18 (d, $J=2.0$ Hz) and 6.38 (d, $J=2.0$ Hz), and 6.19 (d, $J=2.0$ Hz) and 6.38 (d, $J=2.0$ Hz) were assigned to H-6 and H-8, respectively. The B-ring aromatic protons exhibited typical A_2B_2 system at δ 6.92 (2H, d, $J=8.5$ Hz) and 6.88 (2H, dd, $J=2.0, 7.0$ Hz) for H-2' and -6', respectively and δ 8.04 (2H, d, $J=8.5$ Hz) and δ 7.98 (2H, dd, $J=2.0, 7.0$ Hz) for H-3' and -5', respectively, of compounds **4** and **6**. In contrast, the B-ring aromatic



1, R: H
2, R: Glc



3



4, R₁: H, R₂: H
5, R₁: H, R₂: OH
6, R₁: rutinoside, R₂: H
7, R₁: rutinoside, R₂: OH

Fig. 1. Structures of compounds **1 - 7**.

protons exhibited a typical ABX system at δ 7.67 (d, $J=2.0$ Hz) and 7.53 (d, $J=2.1$ Hz) for H-2', δ 6.88 (d, $J=8.5$ Hz) and 6.83 (d, $J=8.0$ Hz) for H-5', δ 7.53 (dd, $J=2.0, 8.5$ Hz) and 7.53 (dd, $J=2.1, 8.0$ Hz) for H-6' of compounds **5** and **7**, respectively. Similar structural signals were observed for compounds **4**, **6** and **5**, **7**. The typical

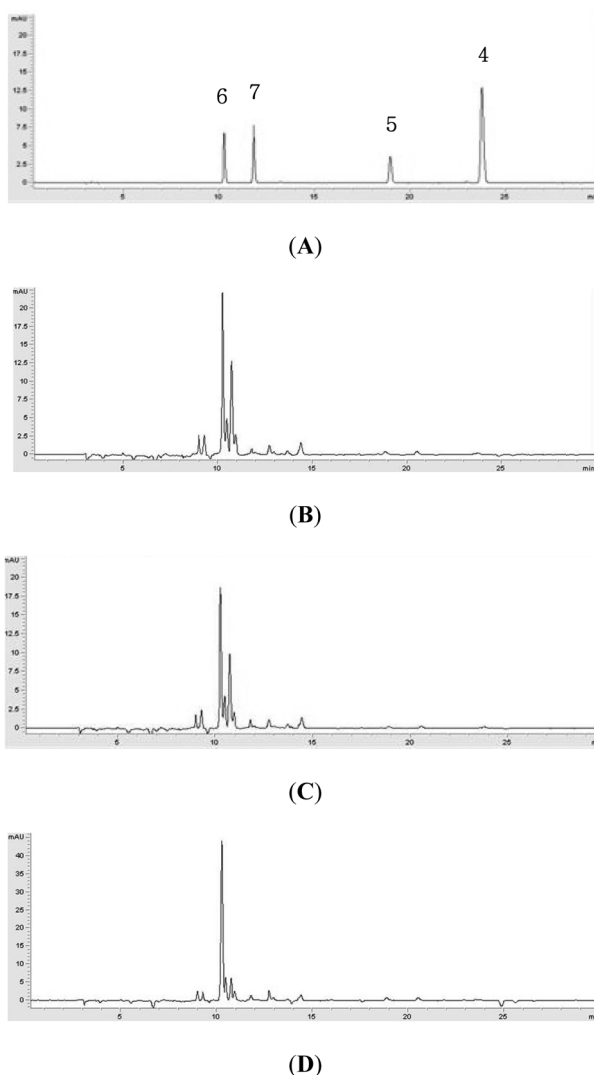


Fig. 2. HPLC chromatograms of compounds **4 - 7** (A), DM (B), YM (C), and TB (D).

pattern of a rutinoside (glucose and rhamnose) moiety was observed in the ^1H - and ^{13}C -NMR spectra in compounds **6** and **7**. The anomeric signal of rutinoside produced a peak at δ 5.30 (d, $J=7.5$ Hz) and 5.34 (d, $J=7.2$ Hz) of glucose and δ 4.38 (d, $J=1.0$ Hz), and 4.38 (br s) of rhamnose. Rutinoside position was at C-3 (β -linkage) of the aglycone according to HMBC analysis. Accordingly, the structures of **4 - 7** were identified as kaempferol, quercetin, kaempferol-3-*O*-rutinoside, and quercetin-3-*O*-rutinoside (rutin), respectively, through a comparison of the spectral data with the literature (Zhang and Luo, 1998; Bao *et al.*, 2003; Zhu *et al.*, 2003).

In particular, quercetin (**5**) and quercetin-3-*O*-rutinoside (**7**) are well-known chemical components of TB, and numerous studies related to the isolation and identification from TB have been published (Jiang *et al.*, 2007; Kim *et*

al., 2008; Dadáková E, and Kalinová, 2010). These compounds from TB have been reported to show antioxidant properties, prevent enzymatic degradation, and have protective effects against lipid peroxidation (Morishita *et al.*, 2007; Jiang *et al.*, 2007; Li *et al.*, 2008).

The HPLC separation of the flavonoids (compounds **4 - 7**) for the simultaneous determination of their identifies was performed using a reversed-phase system with a mobile phase consisting of 0.4% phosphoric acid and acetonitrile (90 : 10 to 80 : 20 for 5 min and then 80 : 20 to 50 : 50 for 35 min). Standard calibration curves for compounds **4 - 7** are shown in Table 3. Compounds **4 - 7** were detected in the seeds of 3 *Fagopyrum* species. Compounds **4 - 7** were detected in the seeds of DM (-, 0.018, 0.033, and 0.450 mg/g, respectively), YM (-, 0.013, 0.032, and 0.266 mg/g, respectively), and TB

(0.007, 0.055, 0.082, and 2.040 mg/g, respectively). In our study, the flavonoid content was the highest in TB (Table 4); in particular, the content of rutin in TB was approximately 4 - 7 times higher than that in the other species. In previous studies, rutin content of TB was found to be higher than that of common buckwheat (Park *et al.*, 2005; Jiang *et al.*, 2007; Kreft *et al.*, 2006).

In conclusion, β -sitosterol (1), β -sitosterol-3-*O*-glucoside (2), oleanolic acid (3), kaempferol (4), quercetin (5), kaempferol-3-*O*-rutinoside (6), and quercetin-3-*O*-rutinoside (7) were isolated from the seeds of TB (Fig. 1). To our knowledge, oleanolic acid (3) has been isolated for the first time from the seeds of *Fagopyrum* species. The concentrations of compounds 4 - 7 in the seeds of *Fagopyrum* species was the highest in TB.

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